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Real-time quantitative PCR for enteric adenovirus serotype 40 in environmental waters

Sunny Jiang, Hojabr Dezfulian, and Weiping Chu

Abstract: Adenoviruses 40 and 41 have been recognized as important etiologic agents of gastroenteritis in children. A real-time PCR method (TaqMan® assay) was developed for rapid quantification of adenovirus 40 (Ad40) by amplifying an 88 bp sequence from the hexon gene. To establish a quantification standard curve, a 1090 bp hexon region of Ad40 was amplified and cloned into the pGEM®-T Vector. A direct correlation was observed between the fluorescence threshold cycle number (Ct) and the starting quantity of Ad40 hexon gene. The quantification was linear over 6-log units and the amplification efficiency averaged greater than 95%. Seeding studies using various environmental matrices (including sterile water, creek water, brackish estuarine water, ocean water, and secondary sewage effluent) suggest that this method is applicable to environmental samples. However, real-time PCR was sensitive to inhibitors present in the environmental samples. Lower efficiency of PCR amplification was found in secondary sewage effluent and creek waters. Application of the method to fecal contaminated waters successfully quantified the presence of Ad40. The sensitivity of the real-time PCR is comparable to the traditional nested PCR assay for environmental samples. In addition, the real-time PCR assay offers the advantage of speed and insensitivity to contamination during PCR set up. The real-time PCR assay developed in this study is suitable for quantitative determination of Ad40 in environmental samples and represents a considerable advancement in pathogen quantification in aquatic environments.

Key words: adenovirus, real-time PCR, environmental waters, serotype 40.

Résumé : Les adénovirus 40 et 41 sont reconnus comme étant des agents étiologiques importants de la gastro-entérite juvénile. Une méthode de PCR en temps réel (essai TaqMan®) a été conçue pour la quantification rapide d'adénovirus 40 (Ad40) par l'amplification d'une séquence de 88 pb provenant du gène de l'hexon. Afin de générer une courbe de concordance de quantification, une région de 1090 pb de l'hexon de Ad40 a été amplifiée et clonée dans le vecteur pGEM®-T. Une corrélation directe fut observée entre la fluorescence du seuil de nombre de cycles et la quantité de départ du gène de l'hexon de Ad40. La quantification était linéaire sur 6 unités logarithmiques et l'efficacité moyenne d'amplification était supérieure à 95 %. Des études d'ensemencement utilisant diverses matrices environnementales incluant de l'eau stérile, de l'eau de ruisseaux, de l'eau saumâtre d'estuaire, de l'eau de mer et des effluents secondaires d'égouts indiquent que cette méthode est applicable à des échantillons environnementaux. Toutefois, le PCR en temps réel était sensible aux inhibiteurs présents dans les échantillons environnementaux. Une efficacité inférieure d'identification par PCR fut notée dans les eaux issues d'effluents d'égouts secondaires et de ruisseaux. L'application de cette méthode à des eaux contaminées par des matières fécales ont pu quantifier avec succès la présence de Ad40. La sensibilité du PCR en temps réel était comparable aux tests traditionnels de PCR nidifié sur les échantillons environnementaux. De plus, le test de PCR en temps réel offre l'avantage de la rapidité et de l'insensibilité à la contamination lors de la préparation du PCR. Le test de PCR en temps réel élaboré dans cette étude est approprié pour le dénombrement de Ad40 dans les échantillons environnementaux et représente un progrès considérable pour la quantification de pathogènes dans des environnements aquatiques.

Mots clés : adénovirus, PCR en temps réel, eaux environnementales, sérotype 40.

[Traduit par la Rédaction]

Introduction

Human enteric viruses represent a diverse group. The term enteric viruses reflect the fact that most of the species

inhabit the alimentary (enteric) tract. Most enteric viruses are non-enveloped RNA viruses with the adenoviridae family being the only enteric viruses containing double stranded DNA genome. Human adenoviruses have been recovered from virtually every human organ and have been associated with a wide spectrum of clinical diseases (Hierholzer 1995). They are also frequently found in urban rivers as well as polluted coastal waters (Puig et al. 1994; Pina et al. 1998; Jiang et al. 2001). There are 51 known serogroups of adenoviruses. Adenovirus 40 (Ad40) and 41 (Ad41) together account for approximately 50% of all types of adenoviruses found in stool specimens (Wigand et al.

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Table 1. Primers and probe for adenovirus 40 hexon gene.

Designation	Function	Sequences	Amplicon size (bp)
f-AD ₁₅₇	Real-time PCR forward primer	5'-ACCCACGATGTAACCACAGACA-3'	88
r-AD ₂₄₅	Real-time PCR reverse primer	5'-ACTTTGTAAGAGTAGGCGGTTTCC-3'	
p-AD ₁₉₆	Real-time PCR probe	6-FAM-CTGCGCTTCGTGCCCCGTCG-TAMRA	
f-AD ₈	Standard control forward primer	5'-CCCCCTCGATGATGCCGCAA-3'	1090
r-AD ₁₀₉₇	Standard control reverse primer	5'-CTGTCCACTGCCTGGTTCCA-3'	

1983; Pina et al. 1998). They are also recognized as one of the major causes of childhood diarrhea.

Recently, adenoviruses have been placed on the US Environmental Protection Agency drinking water Contamination Candidate List (CCL) because they are more resistant to traditional UV disinfection than fecal indicator bacteria during water treatment (Gerba et al. 2002), and therefore have a waterborne disease potential. However, there has not been a method for rapid, sensitive, and accurate detection of the load of adenoviruses in environmental waters. The objective of this study is to develop a real-time PCR method for the quantification of human enteric adenovirus for application in complex environmental matrices. However, because of the genetic variability between Ad40 and Ad41 and the high stringency requirement of real-time PCR, a single assay for the simultaneous detection of both serotypes has not been successful. This report focuses on the development and validation of a real-time PCR method for Ad40 and its application in environmental samples.

Materials and methods

Primers and probe selection

All PCR primers and probes were designed from the human Ad40 hexon gene sequence using Primer Express® Software v. 2.0 (Applied Biosystems, Foster City, Calif.) and are listed in Table 1. Primers and probes were manually selected based on estimated annealing temperature (T_m), the desire for small amplicon size, and the location of the probe and were tested empirically as described below. The specificity of the primer and probe combinations was evaluated using BLAST web software (Altschul et al. 1990). The forward primer has homology with human adenovirus serotypes 40 and 41 and the reverse primer is specific for human adenovirus serotypes 40, 3, 7, and 16. Therefore, the combination of forward and reverse primers is only specific to Ad40. The probe is specific for 17 different serotypes of human adenovirus including Ad 40.

Construction of the real-time PCR standard

A pair of primers (f-AD₈ and r-AD₁₀₉₇, Table 1) targeting at a 1090 bp region of the hexon gene was used to amplify purified Ad40 nucleic acid. The amplicon was subsequently cloned into the pGEM®-T Vector (Promega, Madison, Wis.), transferred into *E. coli* JM109 competent cells, and plated on LB agar containing ampicillin, IPTG (isopropyl- β -D-thiogalactopyranoside), and X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) as described by the manufacturer (Promega). A recombinant plasmid with a single copy of the Ad40 hexon gene insert (designated pAD40) was purified using the Promega Medi-prep Kit following manu-

facturer's instructions (Promega) and quantified spectrophotometrically (Beckman Coulter Inc., Fullerton, Calif.). The copy number of the Ad40 hexon gene was calculated according to the molecular weight of the plasmid. This pAD40 was used as the standard for development of real-time PCR protocols and establishing a standard curve for quantification.

Real-time PCR for the Ad40 hexon gene

To evaluate the efficiency and specificity of the primers, real-time PCR was first conducted using SYBR green nucleic acid dye in an ABI Prism 7000 sequence detection system (Applied Biosystems). The reactions were carried out in a 96-well plate in a 25- μ L reaction volume containing 12.5 μ L of 2 $\times$ SYBR green master mix (Applied Biosystems), a 900 nmol/L concentration of each forward and reverse primer, and pAD40 DNA. The following thermocycling profile was used for each reaction: 50 °C for 2 min to activate uracil *N*-glycosylase in the master mix, 95 °C for 10 min to activate AmpliTaq Gold®, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A dissociation analysis was run at the end of each SYBR green real-time PCR reaction to verify a single PCR amplicon. The size and purity of the amplicons were also examined using conventional agarose gel electrophoresis in some cases.

Real-time PCR with a TaqMan® probe was performed in a 25- μ L reaction mixture with a TaqMan® PCR core reagent (Applied Biosystems). The reaction mixture contained TaqMan® core buffer, 5 mmol/L MgCl₂, 200 μ mol/L each of dATP, dCTP, and dGTP, 400 μ mol/L dUTP, 300 nmol/L of forward primer, 900 nmol/L of reverse primer and 250 nmol/L probe, 0.25 U of AmpErase uracil *N*-glycosylase, 1 U of *Taq* Gold polymerase, and titrated pAD40. The thermalcycling profile was identical to that used for the real-time PCR with SYBR green dye. Each run contained at least 3 no-template controls to establish the baseline emission intensity of the quenched reporter dye.

To achieve optimal real-time PCR settings, primer and probe concentrations were optimized using a concentration matrix spanning a concentration range from 50 to 900 nmol/L for each primer and a concentration range from 50 to 250 nmol/L for the TaqMan® probe. The amounts of target DNA used per reaction varied from 10 to 10⁵ copies to determine the real-time PCR detection limits. Each sample was run in replicate or triplicate. The sensitivity and reproducibility of the reaction were also tested using serially diluted titrated Ad40 viral particles, expanding the range from 10⁻⁴ to 10³ plaque forming units (PFU) per reaction.

A quantification standard curve was generated using serial dilutions of a known copy of pAD40 under optimal PCR conditions determined as described above. A triplicate of

Fig. 1. (a) Relationship between threshold cycle number (Ct) and starting genome copy of the plasmid containing adenovirus hexon gene or diluted adenovirus 40 particles; and (b) relationship between threshold cycle number and plaque forming units of adenovirus 40 titered by the tissue culture infectivity assay. The trend line is fitted using logarithmic regression. The slope of the regression line for purified adenovirus hexon genes and serially diluted adenovirus 40 particles is -1.5519 and -1.5509 , respectively. These slopes are different than those used in the real-time PCR efficiency calculation. Slope for real-time PCR efficiency calculation is based on a linear regression between threshold cycle number and log transformed genome copy.

each dilution was run for each data point, and a standard curve was run for each PCR reaction.

Virus seeding study

Ad40, freshly harvested from tissue culture of human embryonic kidney cell 293A (Goldsmith et al. 1998), was used in the seeding study. For Ad40 purification, the cells with viral infection were harvested in PBS buffer. Adenoviruses were released from infected cells using 3 rounds of freeze-thaw cycles. Cell debris was then removed by low speed centrifugation and viral supernatant was extracted with chloroform. The Ad40 concentration in each batch of harvest was titered by plaque assay on 293A cell line. In brief, serially diluted viral harvest was inoculated onto confluent 293A monolayer cells in 6-well plates. The plates were incubated in a CO₂ incubator at 37 °C for 1 h and were rocked every 15 min for viral adsorption. Inoculated cells were then washed once with pre-warmed calcium and magnesium free PBS (pH 7.4, Mediatech, Inc.) and overlaid with defined minimal essential medium (DMEM) containing 5% fetal bovine serum (FBS) and 1.25% agarose supplemented with 50 µg/mL gentamicin and 2.5 µg/mL amphotericin B. Plates were incubated for 7 days

at 37 °C, 5% CO₂ before plaques were counted under an Olympus Microscope using a 10× magnification lens.

A final concentration of 10⁶ PFU/mL Ad40 was seeded into each of the following environmental matrices: creek water, estuarine water, ocean water, and secondary sewage effluent. Sterilized DI water was used as the reference matrix to determine PCR efficiencies in each type of environmental matrix. All environmental samples were collected in June 2004 using sterilized containers. Creek water was taken from San Diego Creek, a tributary of Newport Bay, Calif. Estuarine water was taken from the middle of Newport Bay, ocean water was taken from Huntington Beach, Calif., and the secondary sewage effluent was obtained from Orange County Sanitation District, Calif.

Viral nucleic acid was extracted from seeded samples using QIAmp Viral RNA Mini Kit (Qiagen, Valencia, Calif.) following manufacturer's recommendation. According to the manufacturer, QIAmp Viral RNA Kit is also efficient at recovery of viral DNA. In comparison, it is more efficient than other blood DNA extraction kits for viral DNA recovery from sewage and other environmental samples where PCR inhibitors may be of concern. Four microlitres of each nucleic acid extract was used in the TaqMan[®] real-time PCR reaction using optimized condition described above.

Assay of environmental samples

To test the feasibility of the real-time PCR method for the detection and quantification of human Ad40 in environmental waters, twelve water samples artificially contaminated with sewage, human feces, dog feces, gull feces, or cattle feces were used as test subjects. These samples were provided to researchers in the lab as "blind samples". Each sample was prepared by mixing 1 to 3 sources of fecal material in sterile water. Ten litres of each water sample was concentrated using a Centrimate Tangential Flow recirculation ultrafiltration system (Pall Life Science, East Hills, New York) with a 30 kDa molecular mass cutoff Omega filtration cassette following the manufacturer's instructions. The concentrates were frozen immediately in aliquots. Viral nucleic acid was purified from concentrates using a QIAamp Viral RNA Mini Kit (Qiagen) and used for adenovirus assay by TaqMan[®] real-time PCR as described above or nested PCR as previously described by Jiang et al. (2001).

Sequence analysis of adenoviral amplicons

Amplicons from nested-PCR were cloned into the pGEM-T vector (Promega) following manufacturer's instructions. Recombinant plasmid with insert was purified and submitted for cycle sequencing using a Dye-Terminator Cycle Sequencing Ready Reaction FS Kit (PE Applied Biosystems) following the manufacturer's instructions. Primers targeted at the T7 and Sp6 promoters were used for bi-directional sequencing. The fragments were trimmed using AssemblyLIGN (Oxford Molecular Group, Campbell, Calif). The final sequences were submitted to a BLAST search of the NCBI GenBank database for identification. ClustalX (Thompson et al. 1997) was used to align sequences. Multiple alignments were performed using default parameters and the final aligned sequences were refined visually. A phylogenetic tree was created using the neighbor-joining method (Saitou and Nei 1987). The branching confidence was estimated by bootstrap

Table 2. Efficiency of real-time PCR detection of adenovirus serotype 40 seeded in different environmental matrices.

Sample matrices	Salinity (ppt)	Genome no. by real-time PCR	Real-time PCR efficiency (%)
Sterile DI water	0	1.6×10^6	100
Secondary sewage	0	5.1×10^5	32
San Diego creek water	0.5	6.7×10^5	42
Newport Bay water	30	9.4×10^5	59
Huntington Beach water	34	9.7×10^5	61

Table 3. Detection of adenoviruses in fecal contaminated water samples by nested and real-time quantification PCR.

Sample No.	Source of contamination	Level of contamination*	Nested PCR	Real-time PCR (genome/100mL)	Serotype†
1	Sewage	0.69	+	320	ND
2	Human and gull feces	0.89 and 0.06	—	0–120‡	
3	Dog and cow feces	3.86 and 96.62	—	—	
4	Human and gull feces	0.22 and 0.24	+	—	40
5	Gull feces	0.30	—	—	
6	Human feces	1.11	—	0–120	
7	Sewage, dog, and cow feces	0.07, 144.9, and 289.9	—	0–120	
8	Sewage	0.69	+	0–120	41
9	Human, dog, and cow feces	0.67, 144.9, and 48.3	—	0–120	
10	Sewage and dog feces	0.14 and 3.86,	+	120	40
11	Cow and gull feces	386.5 and 0.06	—	—	
12	Cow feces	483.1	—	—	

Note: ND, not determined; and —, not detected.

*The level of contamination is given as volume (mL) of sewage or mass (mg) of feces added to per 100 mL of water.

†Serotype is determined by partial sequence of hexon gene.

‡Above threshold cycle number (Ct) was found. However, the Ct value was outside the range of the standard curve (10 to 10^5 copies per reaction), which prevents an accurate estimation of copy number.

analysis using the following parameters: random number generator seed: 111; number of bootstrap trials: 1000. The final tree was viewed in TreeView software. All sequences were submitted to NCBI database under the accession numbers AY747670 to AY747675.

Results

Evaluation and optimization of real-time PCR

Empirical testing of PCR with primers f-AD₁₅₇ and r-AD₂₄₅ using SYBR Green I detector showed a linear increase of SYBR Green I fluorescence intensity with cycle number. Dissociation analysis following PCR yielded a single melting peak at 90.8 °C. This dissociation peak was within 2 °C of the expected T_m, suggesting a specific amplification product (Ririe et al. 1997). A single 88-bp amplicon was also confirmed when the final reaction mixture was visualized on a 2% agarose gel. Therefore, these results suggest that primers f-AD₁₅₇ and r-AD₂₄₅ are capable of specifically amplifying the target with high efficiency. Addition of the probe (without fluorescent dye) did not reduce the amplification efficiency (data not shown), suggesting that there is no interference between the probe and the 2 primers.

Real-time PCR with TaqMan® probe (p-AD₁₉₆) and serially diluted pAD40 DNA yielded a log linear relationship between initial pAD40 copy and threshold cycle number (Ct), spanning a 6-log dilution series (Fig. 1a). Cor-

relation coefficients were always greater than 99%, indicative of accuracy within each experimental run. Slope values varied between –3.25 to –3.57 from run-to-run and averaged –3.40. This translates to an amplification efficiency ($E = (10^{-1/\text{slope}} - 1) \times 100$) of 90% to 103% (average 97%). Optimization of primer and probe concentrations using a concentration matrix indicated that primer concentrations of 300 nmol/L forward and 900 nmol/L reverse, and a probe concentration of 250 nmol/L yielded the lowest threshold cycle (Ct) value. Therefore, this optimized condition was used for all further studies and analyses of environmental samples described below.

The sensitivity of this assay, using pAD40 DNA as targets, yielded a lower detection limit of less than 10 genome copies per reaction (Fig. 1a). The slope value for the amplification of standard DNA insert (pAD40) and titrated Ad40 viral particles was within a margin of less than 0.1, indicating that the efficiencies of the amplification for both types of target were equal (Fig. 1a). The sensitivity of this assay, using titrated Ad40 viral particles as targets, yielded a lower detection limit of 10^{-4} PFU per reaction, equivalent to 2 copies of the hexon gene target (Fig. 1b).

Application in environmental matrices

Table 2 shows the application of this method to various environmental matrices seeded with Ad40. The results indicated that the method is applicable to quantify Ad40

Fig. 2. Phylogenic relationship of partial adenovirus hexon genes retrieved from environmental viral isolates on HEK-293A. Bovine adenovirus 3 partial hexon gene sequence was used as the outgroup. The scale bar indicates 0.1% of sequence divergence. The branching confidence was calculated from 1000 bootstrap trials.

viral concentration in all environmental matrices tested. The viral concentration varied within 1 log unit (3-fold difference between lowest and highest value) in different matrices. However, the efficiency of PCR reduced with the increase of concentration of complex organic material in the sample. Secondary sewage effluent had the lowest PCR efficiency of 32%, whereas ocean water had relatively higher PCR efficiency because of the lower concentration of PCR inhibitors in the sample.

Application of the method to fecal contaminated waters indicated that this method is specific to human fecal source (Table 3). There was no cross reaction with fecal contamination from non-human sources (i.e., dog and gull feces). A positive signal for adenovirus was detected by real-time PCR in samples 1, 2, 6, 7, 8, 9, and 10, which were contaminated with either sewage or human feces. However, the Ct value for samples 2, 6, 7, 8, and 9 fell outside the range of the standard curve (less than 10 copies per reaction), which prevented an accurate quantification of genome copy in the original water samples. Only samples 1 and 10, which were contaminated with 0.69% and 0.14% of sewage, respectively, yielded Ct values within the range of the standard curve. Calculation of the viral genome number in the original water samples after correcting for concentration and extraction factors indicated the concentration of Ad40 detected was proportional to the level of sewage contamination in samples 1 and 10. However, only a weak signal (1 of 2 replicates was above threshold Ct) was detected in sample 8, which was also contaminated with 0.69% of the sewage. Later sequence analysis of adenovirus amplicons from

nested PCR indicated that Ad41 was the dominant serotype presented in sample 8, explaining the low amplification efficiency using Ad40 real-time PCR. Using nested PCR, adenoviruses were detected in samples 1, 4, 8, and 10 (Table 3). All PCR amplicons from the nested-PCR were used for cloning. However, no positive clone was obtained from PCR amplicons of sample 1.

Sequence analysis of a 130-bp Ad40 hexon region recovered from nested PCR indicated that Ad40 and Ad41 were present in the sewage and human feces. The sequences recovered were greater than 95% in similarity to the clinical viral isolates deposited into the NCBI sequence database (Fig. 2).

Discussion

The results of this study demonstrated that the developed primers and probe are capable of efficiently amplifying the Ad40 hexon gene using a real-time fluorescence detector. The real-time PCR is also applicable to environmental samples. The sensitivity of the method is 1000 to 10 000 times greater than that of the plaque assay since the lower detection limit by this method equals 10^{-4} PFU. The assay is rapid. The entire procedure can be completed within 4 h. However, the efficiency of amplification is reduced in presence of PCR inhibitors. Further improvement of nucleic acid purification could improve the efficiency of quantification and detection. Internal seeding of control targets may help to understand the variability of PCR inhibition during field application of this method.

Application of the real-time PCR method to fecal contaminated water samples has demonstrated the specificity of the method. There is no cross-reaction with fecal source of non-human origin. Real-time PCR yields positive signals for 7 of the 8 samples contaminated with sewage and human feces. The negative results may be because of the low concentration of Ad40 in sample 4. Only low signals were detected in samples contaminated with human feces (sample 6). This is likely because of the low level of viruses in the individual human feces used for this study. Sewage from a population of individuals is more likely to contain enteric human pathogens. Compared with nested PCR assay, which detected adenoviruses in 50% of the samples contaminated with sewage and human feces, this single-step real-time PCR is perhaps more sensitive. However, the quantitative ability of this assay is limited only to the samples with relatively higher concentration of viruses. This is in agreement with previous clinical studies comparing single-step real-time PCR with nested PCR (van Elden et al. 2001). Development of nested real-time PCR protocol may further improve the sensitivity of the assay. Nevertheless, the real-time PCR method developed here represents a significant advancement for the rapid quantification of viral load in environmental waters.

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